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Use of Primary Cultures of Salmon Hepatocytes for the Study of Hormonal Regulation of Insulin-like Growth Factor I Expression *In Vitro*

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ABSTRACT—Hepatocytes isolated from sockeye salmon (*Oncorhynchus nerka*) were seeded on an uncoated plastic dish with a positively charged surface, and cultured in hormone-free defined medium. Initially single cells began to aggregate and became flat after being attached to the dish. After approximately 6 days in culture, hepatocytes formed a monolayer sheet with cells in side-by-side contact, and remained in this form for at least 2 weeks. Hepatocytes in multicellular spheroids were also observed. The viability of the cells remained high (85–90%) during culture as judged by trypan blue exclusion. The cells actively incorporated [^{3}H]leucine into cellular and secreted proteins. Salmon and bovine insulins stimulated the leucine incorporation in a dose-dependent manner, suggesting that the cells remained responsive to hormones during culture. The basal IGF-I mRNA level in cultured hepatocytes was much lower than that of *in vivo* status. Salmon GH increased the level of IGF-I mRNA, whereas salmon prolactin had no effect. These findings suggest that the primary culture system of salmon hepatocytes can be used for the *in vitro* study of hormonal regulation of IGF-I gene expression in salmon.

INTRODUCTION

It has been well-documented that insulin-like growth factor I (IGF-I), which is a polypeptide with structure similarity to proinsulin, mediates many of the growth-promoting actions of growth hormone (GH) in mammals [7]. In teleosts, studies concerning the physiological functions of IGF-I has also been reviewed recently [3]. The nucleotide sequences of IGF-I cDNAs have been determined from coho salmon, Atlantic salmon, masu salmon, rainbow trout, and chinook salmon [2, 5, 14, 31, 32]. Mammalian IGF-I is highly active in stimulating cartilage sulfation or body growth in teleost species [10, 11, 15, 23, 24]. IGF-I-like bioactivity in serum and liver extract, and tissue responsiveness to IGF-I have also been found to be GH-dependent in some teleosts [10, 11, 13, 15, 24]. All of these findings strongly suggest that the GH-IGF-I axis is well conserved throughout evolution from teleost fishes to mammals.

In mammals, the major synthesis site of circulating IGF-I is liver, and GH directly stimulates the hepatic IGF-I mRNA expression and IGF-I peptide production [7, 27]. In teleosts, Cao *et al.* [5] reported that injections of bovine GH increased hepatic IGF-I mRNA levels. Duan and Hirano [11] showed that hypophysectomy reduced, and injection of eel GH restored, IGF-I mRNA content in the liver of the eel. They also reported that fasting significantly reduced hepatic IGF-I levels in the eel. Sakamoto *et al.* [30] reported that transfer of rainbow trout from fresh water to seawater increased IGF-I mRNA levels in liver, kidney and gill. In spite of some *in vivo* studies, detailed

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knowledge concerning the regulation of IGF-I production in teleosts is still scarce. To further examine the regulatory mechanism of IGF-I in teleosts, an *in vitro* model has been called for.

Short-term culture of isolated hepatocytes from salmonid species has been extensively explored for their utility in toxicologic and metabolic studies [4, 25]. Long-term culture of salmon or trout hepatocytes, however, has not been studied as extensively. The purpose of the present study was to establish a long-term primary culture system of salmon hepatocytes and to examine the effect of GH on IGF-I mRNA expression *in vitro*.

MATERIALS AND METHODS

Animals

Sockey salmon (350–600 g body weight) were obtained from Sunlock, Ltd., Kamaishi, Iwate. The fish were held in seawater in a flow-through 1,000 *l* aquarium and maintained at 15–17°C under natural photoperiod. They were fed once per day with commercial pellets.

Hormones

Bovine insulin (24 U/mg) was purchased from Sigma. Salmon insulin was provided by Dr. Erika Plisetskaya, University of Washington, Seattle, WA. Recombinant chum salmon GH was provided by Kyowa Hakko Kogyo, Co, Ltd.,Tokyo, and chum salmon prolactin was a gift from Dr. Hiroshi Kawauchi, Kitasato University, Sanriku, Iwate, Japan.

Isolation of hepatocytes

Fish were anesthetized by immersion in 0.02% MS222 prior to handling. A polyethylene cannula, connected to the perfusion reservoir, was inserted into the intestinal vein. The liver was perfused with an initial flow rate of 2 ml/min with 30 ml Ca^{2+}-free Krebs-Ringer. Then, the liver was perfused with 50 ml of the perfusion medium containing collagenase (Wako Pure Chemical Industries, 0.3 mg/l) for 25 to 30 min. The liver was removed from the fish and minced with scissors. The minced tissue was transferred to a beaker with 50 ml Ca^{2+}-free Krebs-Ringer containing collagenase, and mixed by stirring with a magnetic bar for 30 min. The suspension was filtered through a filter unit (150-mesh screen) and centrifuged at 50 $\times g$ for 2 min. The supernatant was discarded and the cells were resuspended in Krebs-Ringer and centrifuged for 2 min. The washings were repeated three more times. The final pellet was suspended with culture medium to a volume of 20 ml. Cells were counted in a Thoma haemacytometer.

Cell culture

Cells were suspended in William's E medium (Sigma) with 5% of fetal bovine serum (Sigma) and plated at 3×10^6 cells/60-mm Falcon Primaria dish. After about 12 hr, non-adherent cells were discarded, and the medium was replaced with serum-free William's E medium after washing 3 times by Dulbecco's phophate buffered saline (PBS). The cells were incubated in 5%CO_2/95% air at 15°C. Cells cultured for 5 or 6 days were used for the experiments.

Cell morphology

Morphology of the cells was examined by phase-contrast microscope and scanning electron microscope. For scanning electron microscope, the cells were fixed with 1% glutaraldehyde, postfixed with 1% OsO_4, dehydrated through a graded series of ethanol, and finally soaked in isoamyl acetate. The cells on the plate were dried with a critical point dryer (Hitachi, HPC-1), coated with Pt-Pd using an Eiko Ion Coater, and then observed with a JSM-U3 scanning electron microscope.

Incorporation of [^{3}H]leucine

After washing with PBS without Ca^{2+} and Mg^{2+} 3 times, the cells were incubated in 2 ml fresh William's E medium with or without hormone for 16 hr. Then, 2 μCi [^{3}H] leucine (NEN) was added and incubated for 4 hr. Four to five dishes/group were used in each treatment. The measurement of incorporation of [^{3}H]leucine into hepatocyte as well as into the secreted proteins was performed as described by Plisetskaya *et al.* [28] with some modification. Briefly, the cells were separated from the medium by centrifugation for 5 min at 300$\times$g. The cell pellets were resuspended in ice-cold water and sonicated. The proteins from

both the sonicated cells and the medium were precipitated by ice-cold 5% trichloroacetic acid (TCA). The TCA precipitates were washed twice with 5% TCA. Then, the pellets were dissolved in 1 ml 0.1 M NaOH. The radioactivity was measured in a scintillation counter (Packard TRI-CARB 300) after adding 6 ml scintillation fluid (Aquasol-2, NEN). The radioactivity in the cell fraction was expressed as intracellular protein, and that in medium fraction was expressed as extracellular or secreted protein. The results were presented as DPM/10^6 cells viable at the beginning of the tracer experiment.

RNA isolation and Northern blot analysis

The hepatocytes cultured for 6 days were incubated in 2 ml fresh William's E medium with or without hormone for 24 hr. After removal of the culture medium, cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS. Total RNA was isolated with a single-step extraction method using acid guanidium thiocyanate [6]. Poly [A^+] RNA was purified by oligo(dT)-latex using a commercial kit (Oligotex-dT30, Takara). A 1.1 kb cDNA probe encoding the entire coding sequence for coho salmon IGF-I (provided by Dr. Stephen J. Duguay, University of Washington, Seattle, WA) and a chicken β-actin cDNA probe (Sigma) were labelled with [^{32}P] by a multiprime DNA labelling kit (Amersham).

Poly [A^+] RNA was denatured by 2.2 M formaldehyde, electrophoresed on 1% agarose gels, and bound to a nylon filter (Hybond^{TM}) by capillary transfer in 20×SSC. The hybridization was performed as previously described [11]. Autoradiography was performed by exposing the filters to Kodak X-Omat AR film at −70°C.

Statistical analysis

The results were expressed as means±SE, and differences among groups were evaluated statistically by ANOVA followed by the Fisher Protected Least Significant Difference Test (PLSD) using the Statview 512+ [8].

RESULTS

Changes in hepatocytes morphology during primary culture

The yield of liver cells obtained by the present method was about 1×10^8 cells/g liver. Parenchymal cells (hepatocytes) were the major cell type (more than 98%). The viability was usually greater than 90% as judged by trypan blue exclusion test. Figure 1a shows the typical spherical shape of freshly isolated salmon hepatocytes. Isolated salmon hepatocytes were single, round and large cells.

The attachment of salmon hepatocytes was incomplete in ordinary non-coated culture dishes. The plating efficiency in these ordinally non-coated culture dishes was usually lower than 20%. Preliminary experiments showed that coating the dish with bovine fibronectin improved the attachment to some extent, but bovine collagen or gelatin was ineffective (data not shown). Subsequent results showed that salmon hepatocytes attached firmly to Falcon Primaria culture dishes (Fig. 1b), which have positively charged surface, after overnight incubation (plate efficiency higher than 85%). Thus, cells were cultured in Primaria culture dishes in subsequent experiments.

Salmon hepatocytes showed significant morphological changes after attachment to the dish surface. Initially, single, round hepatocytes began to aggregate to each other, and became flat in 3–5 days (Fig. 1b). After being cultured for 6 days, cells spread and formed typical monolayer (Fig. 1c). The boundaries between cells became clear, and nuclei were clearly observed. They remained in this form for up to 3 weeks. Thereafter, they began to die, and more fibroblast-like cells appeared in the culture dishes (Fig. 1d). The multicellular spheroids with highly packed cells were observed sometimes (Fig. 2 a, b).

Protein synthesis and effect of insulin

Cultured hepatocytes actively incorporated [^{3}H] leucine into intracellular and extracellular proteins. Actinomycin D, which is an inhibitor of RNA synthesis and can affect protein synthesis via inhibition of RNA synthesis, decreased the [^{3}H]

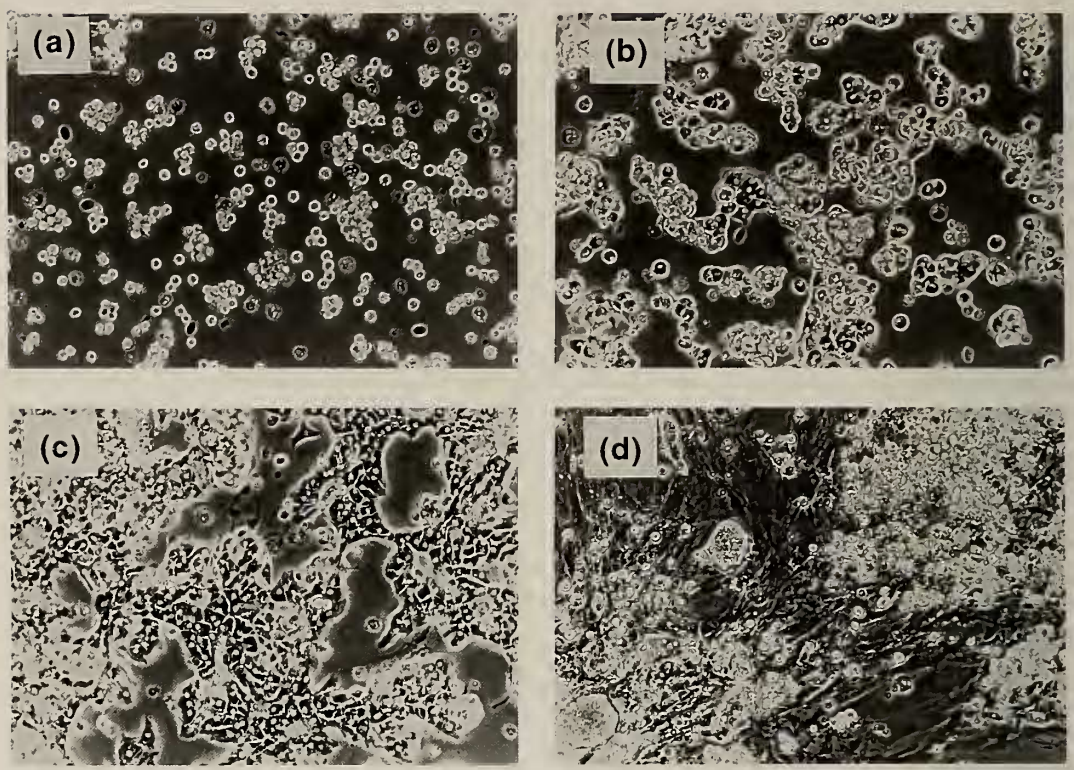

FIG. 1. Phase-contrast microscopy of salmon hepatocytes in primary culture. Cells at day 0 (a); day 3 (b); day 10 (c); day 30 (d). All magnification are ×200.

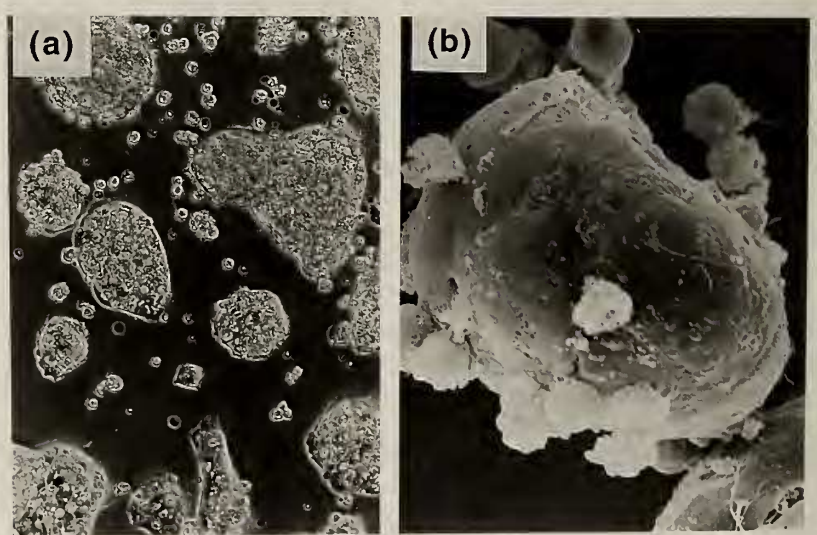

FIG. 2. Multicellular spheroids of salmon hepatocytes. (a) Phase-contrast microscopy (magnification, ×200); (b) scanning electron microscopy (magnification, ×1,000).

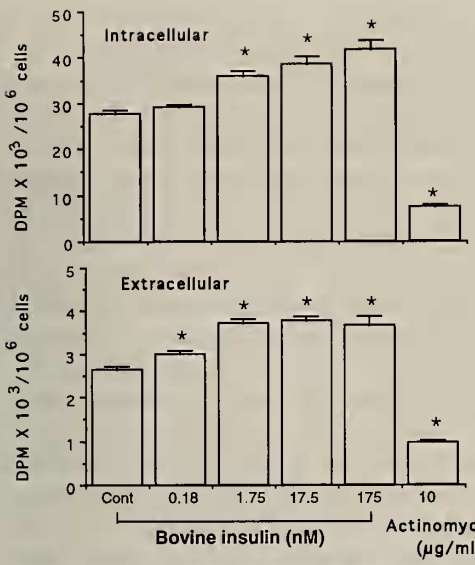

FIG. 3. Effect of insulin on the incorporation of [^{3}H] leucine into intracellular and extracellular proteins in primary cultures of salmon hepatocytes. Data are means ± SE (n=5). *Significantly different from the control ($p<0.05$).

leucine incorporation into proteins in a dose-dependent manner. At a dose of 10 μg/ml, it significantly reduced the incorporation of labelled leucine into intracellular and extracellular proteins (26 and 36% of the control levels, respectively) (Fig. 3), indicating that most of the [^{3}H] leucine measured was indeed incoporated into newly synthesized proteins in this system.

As shown in Figure 3, bovine insulin stimulated the [^{3}H] leucine incorporation into both intracellular proteins and extracellular proteins in a dose-dependent manner. The minimum effective dose was 1.75 nM for intracellular protein synthesis, and 0.18 nM for protein secretion. Similar dose-dependent stimulation was also obtained with homologous salmon insulin (data not shown).

IGF-I mRNA expression and effect of GH

Figure 4 shows the results of a Northern blot with 15 μg of poly [A^+] RNA isolated from intact salmon liver or hepatocytes cultured without any hormone for 6 days. Both liver and hepatocyte RNA showed a major class of IGF-I mRNA (approximately at 3.9 kb). The signal of hepatocyte RNA was much weaker than liver RNA so

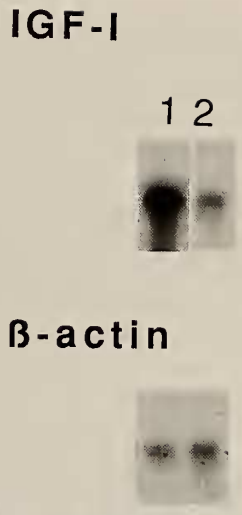

FIG. 4. Northern blot analysis of RNA. Fifteen micrograms of poly [A^+] RNA isolated from salmon liver (land 1) and six-day-old cultured salmon hepatocytes (lane 2) were used. (a) IGF-I mRNA. The exposure time was 2 days for liver RNA (lane 1) and 7 days for hepatocyte RNA (lane 2). (b) β-Actin mRNA. The same blot was rehybridized with chicken β-actin cDNA. The exposure time was 2 days.

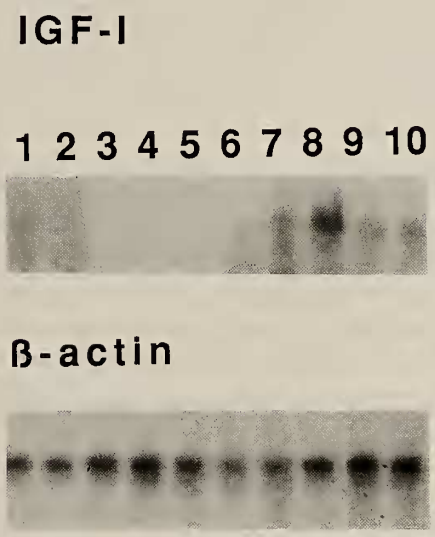

FIG. 5. Effects of salmon GH and prolactin on IGF-I mRNA levels in primary cultures of salmon hepatocytes. Lanes 1–2: prolactin 1,000 ng/ml; lanes 3–4: prolactin 100 ng/ml; lanes 5–6: control; lanes 7–8: GH 100 ng/ml; lanes 9–10: GH 10 ng/ml. Approximately two microgram of poly [A^+] RNA was used for each lane. The exposure time was 7 days for both IGF-I and β-actin mRNA.

that the blot had to be exposed for longer time (7 days as compared to 2 days of liver RNA), suggesting low expression level of IGF-I mRNA in hormone-free culture condition *in vitro*.

The effect of chum salmon GH and prolactin on IGF-I mRNA expression was then examined in primary cultures of salmon hepatocytes. Approximately 2 μg poly [A^+] RNA were obtained from each dish, and used for Northern blot analysis. Clear hybridization signals of IGF-I mRNA were obtained in GH-treated group (Fig. 5a, lanes 7–8, GH 100 ng/ml), whereas no apparent hybridization signal of IGF-I mRNA was seen in the control group or prolactin-treated groups after 7 days of exposure (Fig. 5a, lanes 1–6). When rehybridized the same blot with a β-actin cDNA probe, similar levels of clear β-actin mRNA signal were obtained with all groups (Fig. 5b, lanes 1–10).

DISCUSSION

In this study, salmon hepatocytes were cultured in a serum-free medium for up to several weeks. The cultured cells were responsive to insulins in term of stimulation of protein synthesis. GH stimulated IGF-I mRNA expression in primary cultures of hepatocytes, whereas prolactin had no effect.

There are many reports on culture of fish hepatocytes [25, 4]. To date, most of the studies used freshly isolated liver cells in suspension or primary culture cells over very short periods (within 24 h), whereas few studies were carried out with long-term primary culture system in a defined medium. This is in sharp contrast to the extensive studies on long-term cultures of mammalian hepatocytes [26]. This was partly because the attachment of fish hepatocytes to the surface of culture plate was slow and remained relatively weak, making it difficult to manipulate the cultures without disrupting of the cells [17, 22]. To solve this problem, efforts have been made to coat the culture plate various extracellular matrix components such as mammalian fibronectin, collagen, gelatin and fish serum or fish skin extract [4, 16, 18, 20, 22]. While some of the matrix components such as mammalian and fish fibronectins improved the efficacy of attachment [18], it is time-consumimg to prepare the coated plates. In this study, we have shown that salmon hepatocytes attached to Primaria dishes firmly without any treatment, and survived well up to several weeks.

The freshly isolated salmon hepatocytes seemed undamaged when viewed under phase-contrast microscope and scanning electron microscope (data not shown). The viability of the cells was approximately 90%. During the culture, cells reaggregated, became flat, and spread to form a monolayer. While most cells were in the monolayer form, some spheroids were also observed. In mammals, two distinct features of primary culture of adult hepatocytes are observed when the cells are cultured in Primaria dishes: monolayer culture with proliferative activity and multicellular spheroids with poor proliferative activity and high albumin-producing ability [21]. It is not clear whether the biochemical and physiological properties of these two forms of salmon hepatocytes in culture are similar to those of mammalian hepatocytes.

It has been well documented in teleosts that insulin stimulates protein synthesis both *in vivo* and *in vitro* [1, 19, 28]. In the present study, a dose-dependent stimulation was obtained with mammalian and salmon insulins. The minimum effective dose was 1.75 nM, which is within the physiological range of circulating insulin salmonids [29]. The dose dependency and minimum effective dose of insulins are consistent with previous findings obtained in intact animals, cultured liver slices, and suspension of isolated liver cells [1, 19, 28], suggesting our primary culture system is suitable for study of hormone action.

Although some *in vivo* studies have indicated the GH-regulated hepatic IGF-I mRNA expression in teleosts [5, 11, 9, 30], there was no published *in vitro* information to date. In the present study, Northern blot analysis revealed a major band of 4 kb with cultured hepatocyte RNA, which was identical to that obtained with intact liver RNA. A longer exposure time, however, was necessary to visualize the hybridization signals with hepatocyte RNA (7 days) than with liver RNA (2 days), indicating a lower level of IGF-I mRNA expression *in vitro* as compared to that of *in vivo*. The lower expression level is likely due to the lack of

hormone stimulation, because the cells were cultured in hormone-free medium. Addition of salmon GH to the medium at a concentration of 100 ng/ml markedly increased the IGF-I mRNA levels. There was no apparent signal in the control and prolactin-treated groups. Since β-actin mRNA levels were all similar, the absence of IGF-I mRNA in the control and prolactin-treated groups is most likely to be due to low expression levels of IGF-I mRNA. These results suggest that GH has a direct effect on hepatic IGF-I mRNA expression in salmonid species, and that our primary culture system can be used as an *in vitro* model to study the hormonal regulation of IGF-I expression.

While our study demonstrated for the first time that GH stimulates hepatic IGF-I mRNA expression *in vitro* in a teleost species, the accurate quantitation of the results was difficult due to the low sensitivity of Northern blot and the low expression level of IGF-I. As mentioned above, using Northern blot, we were unable to detect IGF-I mRNA in poly [A^+] RNA (2 μg) prepared from hepatocytes of the control groups even after a long exposure unless using larger amount of RNA sample. Thus, a more sensitive and reliable assay is necessary for further study of hormonal regulation of IGF-I mRNA expression *in vitro* in salmonids. RNA dot blot or slot blot is a simpler version of Northern blot technique, and is slightly more sensitive and semi-quantitative. However, results of dot-blot or slot blot can be misinterpreted if the RNA samples are contaminated by DNA. In comparison, another technique, solution hybridization/RNase protection assay, is more sensitive, specific and fully quantitative. Recently, we have developed a solution hybridization/RNase protection assay for salmon IGF-I mRNA [9]. Quantitative data obtained by this assay confirmed the finding of the present study: GH but not prolactin stimulates IGF-I mRNA expression in primary cultures of salmon hepatocytes. Studies concerning the detailed regulation mechanism of IGF-I mRNA expression in salmon are in progress using the primary culture system of hepatocytes established in the present study.

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